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Gene expression analysis of ELF-MF exposed human monocytes indicating the involvement of the alternative activation pathway

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Abstract

This study focused on the cell activating capacity of extremely low frequency magnetic fields (ELF-MF) on human umbilical cord blood-derived monocytes. Our results confirm the previous findings of cell activating capacity of ELF-MF (1.0 mT) in human monocytes, which was detected as an increased ROS release. Furthermore, gene expression profiling (whole-genome cDNA array Human Unigene RZPD-2) was performed to achieve a comprehensive view of involved genes during the cell activation process after 45 min ELF-MF exposure. Our results indicate the alteration of 986 genes involved in metabolism, cellular physiological processes, signal transduction and immune response. Significant regulations could be analyzed for 5 genes (expression >2- or <0.5-fold): IL15RA (Interleukin 15 receptor, alpha chain), EPS15R (Epidermal growth factor receptor pathway substrate 15 — like 1), DNMT3A (Hypothetical protein MGC16121), DNMT3A (DNA (cytosine-5) methyltransferase 3 alpha), and one gene with no match to known genes, DKFZP586J1624. Real-time RT-PCR analysis of the kinetic of the expression of IL15RA, and IL10RA during 45 min ELF-MF exposure indicates the regulation of cell activation via the alternative pathway, whereas the delayed gene expression of FOS, IL2RA and the melatonin synthesizing enzyme HIOMT suggests the suppression of inflammatory processes. Accordingly, we suggest that ELF-MF activates human monocytes via the alternative pathway.

Keywords: Gene expression; Monocyte; Cytokine receptor; Early response gene; ELF-MF or electromagnetic field; Free radical; Alternative activation pathway

Abbreviations: $.O_2^-$, super oxide anion; AML, acute monocytic leukaemia; as, antisense; C.I., confidence interval; C3, complement component 3; CD, cluster of differentiation; CP, crossing point; DHR, dihydrorhodamine 123; E/C, ration of exposed to unexposed cells; ECM, extracellular matrix; ELF-MF, extremely low frequency magnetic fields; FITC, Fluorescein; GTP, guanosine triphosphate; HBS, HEPES-buffered saline; HIOMT, hydroxyndole-*O*-methyltransferase; HPRT, hypoxantine phosphoribosyl-transferase1; HUPO, human acidic ribosomal protein; IL, interleukin; IL10RA, interleukin 10 receptor alpha; IL15RA, interleukin 15 receptor alpha; IL2RA, interleukin 2 receptor alpha; JAK, janus kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated phosphatase kinase; NADH, reduced NAD; NADPH, NAD phosphate (reduced); PDGFA, platelet-derived growth factor alpha polypeptide; PerCP, Peridinin–Chlorophyll–Protein-Complex; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute; RT-PCR, reverse transcriptase polymerase chain reaction; s, sense; SAM method, statistical analysis of microarray; S.D., standard deviation; VEGF, vascular epidermal growth factor

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1. Introduction

In previous studies, we showed the activating capacity of extremely low frequency magnetic fields (ELF-MF) (50 Hz) in murine macrophages [1,2], human umbilical cord blood-derived monocytes and human acute monocytic leukaemia (AML) cell line Mono Mac 6 [3]. We detected an increased production of free radicals after 45 min ELF-MF exposure (1.0 mT), indicating the activation of immune relevant cells, which was mediated by the NADH-dependent pathway in murine macrophages and in Mono Mac 6 cells, but by the NADPH-dependent pathway in human monocytes. Both represent major pathways in immune cells for the formation of the superoxide radical anion ($^{\cdot}O_2^-$), the precursor of other reactive oxygen species (ROS). The generation of ROS is essential to maintain homeostasis and constitutes an essential host defence mechanism in monocytes. It is also necessary to combat infections [4] by actively participating in physiological process, such as cell growth, apoptosis, cellular senescence [5], induction of intracellular signal transduction pathways [6], regulation of gene transcription and response to stress [7]. It is known that signal transduction from various membrane receptors, e.g., cytokine receptors, is enhanced by ROS by triggering concomitantly the activation of NAD(P)H pathways followed by corresponding downstream signalling pathways [8]. The activation of downstream pathways causes a change in gene expression mainly by the modulation of the activities of transcription factors [4,9].

It has been suggested that ELF-MF induces immune cell activation [1–3,10,11], but the causal mechanism is not clear yet. For immune cell activation, three different pathways are described: the classical, the alternative, and the lectin-dependent activation pathway. The classical activation pathway includes the activation of inflammation, the destruction of the extracellular matrix (ECM), and the induction of apoptosis. The alternative activation pathway promotes ECM construction, cell proliferation, and angiogenesis, and resolves inflammation. The lectin pathway initiates inflammation, apoptosis and inhibits cell growth in a comparable way to the classical activation complex [12–15].

In this study, we used human umbilical cord blood-derived monocytes positively responding to 1.0 mT 50 Hz magnetic fields (45 min) with ROS production and applied a genome wide gene expression analysis as a discovery science approach to detect gene regulations after ELF-MF exposure. Changes in a number of genes allowed us to formulate a working hypothesis, namely the activation of the alternative pathway by ELF-MF, which was tested by specific gene analysis using real time RT-PCR. The expression of IL10RA, IL15RA, IL2RA, FOS, and the melatonin synthesizing enzyme hydroxyndole-*O*-methyltransferase (HIOMT) was investigated after 5, 15, 30 and 45 min ELF-MF exposure, to obtain specific information about the involved pathway. Interleukin receptor 15 (IL15RA) was investigated, because it was significantly upregulated according to the gene expression profiling. IL2RA was examined since both IL15RA and IL2RA elicit similar biological effects indicating pro-inflammatory responses [16,17]. Furthermore IL10RA was investigated to examine the induction of anti-inflammatory response by ELF-MF. Since HIOMT is one key enzyme for melatonin synthesis, and it is also responding to inflammation by upregulating IL-2 production [18], the time-dependent regulation of the HIOMT gene was analyzed as well. Finally, the immediate early response gene FOS, acting as a transcription factor, was analyzed, since it is reported [19,21] to be affected by ELF-MF.

2. Material and methods

2.1. Cell culture

Human umbilical cord blood monocytes were derived from the maternal end of the severed cord immediately after the birth and isolated as previously described [3] within 24 h. Briefly, after the standard density gradient Ficoll hypaque separation (density =1.077, PAA, Germany), monocytes were separated from mononuclear cells using the $CD14^+$ Cell Isolation Kit and the MiniMacs separation unit (Miltenyi Biotec GmbH, Germany). Cells were counted using a Neubauer cell count chamber and seeded at a density of $0.5–1.0 \times 10^6$ cells/ml in RPMI 1640 containing 2 mM L-glutamine (PAA, Germany), and 10% autologous serum [22]. Cells were cultured at 37 °C in a humified atmosphere and 5% CO_2 until the experiments started.

2.2. Flow cytometric analysis of human monocytes

Characterization of human monocytes was evaluated by double-stained labelling of 0.5×10^6 cells with anti-CD14-FITC and anti-CD69-PerCP antibody (Becton Dickinson, Germany), representing monocytes ($CD14^+$) in an early activation stage ($CD69^+$). A total of 5000 cells were analyzed per sample by using the flow cytometer Epics Altra (Beckman Coulter, Germany) with a water - cooled 488 nm Argon Laser. Fluorescence values were measured at 525 nm (FITC conjugated antibodies) and at 610 nm (PerCP conjugated antibodies). The percentage of $CD14^+/CD69^+$ and $CD14^+/CD69^-$ cells were calculated by using the software EXPO32 vs. 1.2 analysis (Beckman Coulter, Germany).

2.3. Exposure system

The ELF-MF exposure system consisting of a Helmholtz coil with a diameter of 400 mm, a distance between the coils of 200 mm, a resistance of 2.1 Ω and 154 turns of copper wire (Phywe Systeme, Germany) [23], was located inside a CO_2 - incubator (Binder, Germany) at 37 °C with a humified atmosphere and 5% CO_2. Cells were exposed in the central area of the coils to 50 Hz ELF-MF to 1.0 mT flux density continuously monitored by precision F.W. Bell Model 4048 Gauss/Tesla Meter (Bell, Orlando, USA). Control cells were cultured in an identical incubator without a Helmholtz coil system in parallel.

2.4. Detection of reactive oxygen species

The production of reactive oxygen species was detected by using the dye dihydrorhodamine 123 (DHR, Molecular Probes, The Netherlands), which will be oxidized by ROS to the fluorescence dye rhodamine. Cells were seeded in 96-well plates with flat bottom (TPP, Germany) at a density of 1×10^6/well and treated with 1 μM PMA (Sigma Aldrich, Germany) or 1 μg/ml LPS (Sigma Aldrich, Germany), or exposed to ELF-MF for 45 min. After exposure, cells were centrifuged and HBS including DHR solution (1 μM final concentration) was added for 25 min as previously described [3]. The fluorescence of rhodamine was measured by flow cytometry (Epics Altra, Beckman Coulter, Germany) at 525 nm. Mean fluorescence intensity (X-mean) was determined after gating monocytes by using the software EXPO32 vs. 1.2 analysis (Beckman Coulter, Germany). For each sample 2500 events were acquired.

Data for 45 min exposure are expressed as ratio of exposed to respective control cells (E/C), whereas data of the time dependency are expressed as the ratio of exposed to control cells at $t=0$ min (E/C_{t0}). Columns show the arithmetic mean ratio±standard deviation (E/C±S.D.) of the mentioned (n) independent experiments. Each measurement was carried out in triplicates.

2.5. RNA preparation

Total cellular RNA was isolated from unexposed and ELF-MF exposed monocytes by using the RNeasy Mini Kit (Qiagen, Germany). To collect the appropriate amount of RNA for the gene expression profiling, pooled RNA was used from 28 independent experiments of 74 donors. For real-time PCR, RNA was pooled from 5 and 6 donors and was used in 2 independent experiments. The quality of all isolated RNA samples was determined by a GeneQuant Analyzer (Pharmacia, Germany).

2.6. Human cDNA array

The cDNA array profiling was performed with RNA derived from unexposed and ELF-MF exposed monocytes. Total RNA was pooled and concentrated using the RNA MinElute Kit (Qiagen, Germany). RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies).

For the cDNA array, 6.5 ng of RNA were used for labelling with α-CTP (^{33}P). Samples were added to the whole-genome cDNA array Human Unigene RZPD-2 with 75.000 clones for a competitive hybridization (65 °C for 17 h). Each array contains 2 spots for each clone. Since the experiments were performed in duplicate, there were 4 spots potentially available for each clone. At the end, the cDNA arrays were scanned using a phosphorimager (Fuji FLA-3000). Background correction and normalized signal medium intensities were extracted using Aida Version 3.23 software. The software Array Vision analyzed the ratio of exposed to unexposed cells. Ratios of ≤0.5 and ≥2.0 were taken into consideration, if at least 3 of 4 spots were appropriate.

2.7. Two step real-time RT-PCR

RNA was isolated from unexposed and ELF-MF exposed monocytes after 0, 5, 15, 30 and 45 min in 2 independent experiments to determine time-dependent gene regulation.

cDNA used for real-time PCR amplification was first synthesized from 150 ng total cellular RNA using 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) using oligo (dT) primer (Roche, Germany). PCR amplification was performed using the LightCycler DNA Master SYBR Green I (Roche, Germany) according to the manufacturer's protocol. The following transcript specific oligonucleotide primers (Invitrogen, Germany), were used for PCR amplification (LightCycler, Roche, Germany): IL2RA (197 bp, exon 4–5): (s) atcagtgcgtccagggatac (as)gacgaggcaggaagtctcac; IL10RA (226 bp, exon 6–7): (s)ttctttgcctttgtcctgct (as)gcaggtccaagttcttcagc; IL15RA (213 bp, exon 4–6): (s) accttccacaggaaccacag (as)aggtagcatgccaggagaga; HIOMT (251 bp, exon 3–6): (s)tctgtgtgtccctgaagctg (as)ggacctgtagatggccgtaa; FOS (366 bp, exon 1–3): (s) ccggggatagcctctcttac (as)cccttcggattctccttttc; HPRT (345 bp, exon 3–6): (s) acgtcttgctcgagatgtga (as)cttgcgaccttgaccatctt.

Crossing points (CP) values were obtained from the LightCycler software Version 3.5, representing the PCR cycle at which an increase in fluorescence intensity above a base line signal was first detected. Relative quantification of the target gene signal was calculated by normalizing the target gene signal with the hypoxanthine phosphoribosyl-transferase1 (HPRT) signal, whereas normalization to β-actin or human acidic ribosomal protein (HUPO) did not yield acceptable results.

The ratio of exposed to unexposed genes was calculated by the following formula [24]:

$$\text{Ratio } (E/C) = 2^{-[\Delta\text{CP sample}-\Delta\text{CP control}]} = \text{Ratio } (E/C) = 2^{-\Delta\Delta\text{CP}} \quad \left(\Delta\text{CP} = \text{CP}_{\text{HPRT}} - \text{CP}_{\text{target gene}}\right)$$

Ratios for time dependency values were calculated to control values at $t=0$ min. Results are expressed as the mean ratio±standard deviation (E/C±S.D.) of 4 data points obtained from 2 independent experiments.

2.7.1. Primer specificity

Specificity of real-time PCR products was documented with high resolution gel electrophoresis and resulted in a single product with the desired length. Additionally, melting curve analysis was performed after real-time PCR amplification to determine the characteristic melting temperature of the target cDNA. The melting temperature supplies information for product identification and unwanted by-products (primer dimers). Analysis was accomplished using the melting curve analysis tool of the LightCycler Software. Furthermore, PCR products of each gene were pooled, run by gel electrophoresis, extracted using the gel extraction kit (Qiagen, Germany) and sequenced.

2.8. Statistical analysis

Statistical analysis of ROS production was performed by using the one or two way ANOVA and between investigated groups the Student's t test, considering values $P<0.05$ as significantly different. The SAM method [25] was applied for statistical analysis for the gene expression profiling considering clones with 4 good spots (38581 over 74 543 total clones corresponding to 51.8% ratio). The normalized difference diff(i) between rescaled values for each clone i was calculated according to the following formula:

$$\text{diff}(i) = \frac{E[\exp(i)] - E[\text{ctrl}(i)]}{\sqrt{\frac{\sigma_{\text{ctrl}(i)}^2}{\text{NG}-1} + \frac{\sigma_{\exp(i)}^2}{\text{NG}-1}} + s_0},$$

where exp(i) denotes the 4 spots related to the ith clone of the exposed cells, ctrl(i) denotes the 4 spots related to the ith clone of the control cells, $E[\ ]$ is for "expected value of", and s_0 is an additional correction term that removes possible divergences in the denominator, calculated as the median of the σ_i distribution for each clone. Genes are ranked by their diff(i) value – rank[diff(i)] – and such ranking is compared with a "null" distribution (corresponding to no EMF effect) obtained with a bootstrapping [26] procedure from the dataset. Namely, $P=36$ new datasets are generated by means of permutations of the original data (mixing ctrl and exp values), and $\text{diff}_p(i)$ (p referring to the pth permutation) was calculated for these datasets. These differences were ranked for each dataset – rank[$\text{diff}_p(i)$] – and the average rank E[rank[$\text{diff}_p(i)$]] for each gene over all the permutations was calculated. A plot of rank [diff(i)] versus $E\text{diff}_p(i)\text{diff}_p(i)$] was generated, and the genes were chosen as those that significantly deviated from the $y=x$ line: the distance of rank[diff(i)] from the $y=x$ line, defined as dist(i), was to be larger than a threshold value $\Delta=1.5$.

3. Results

3.1. Characterization of human umbilical cord blood derived monocytes

The cell surface antigen CD14 is continuously expressed on monocytes, whereas CD69 is expressed only at the early activation stage. This characterization was necessary to show the activation potency of the used cells and was performed before all experimental applications. Flow cytometric analysis detected an enrichment of 90–98% $CD14^+$ monocytes, whereas ca. 30% of these cells expressed CD69 (data not shown).

Determination of ROS production was performed to detect the cell activating capacity of ELF-MF in human monocytes [3]. PMA treatment was carried out for ROS production as positive control for cell activation [25], and only positively responding

cells were used for all experiments. Monocytes, used for the gene expression profiling, showed a 1.5-fold significantly increased ROS release after 45 min ELF-MF exposure (1.0 mT) and a 4.3-fold increase after PMA treatment (Fig 1).

3.2. Time-dependent ROS production in human monocytes

Time-dependent ROS production was investigated in three independent experiments of 2, 4, and 7 donors to obtain information about the magnitude of activation capacity of ELF-MF within 45-min exposure in human monocytes. PMA treatment stimulates ROS production via a receptor independent [25] and LPS via a receptor-dependent pathway [27], which were used as positive controls.

ELF-MF induced a 1.4-fold increased ROS production already after 5 min ELF-MF exposure, which remained constant over the observed time period (Fig. 2). LPS treatment caused a significantly increased ROS production at the same magnitude as ELF-MF exposure, whereas the PMA induced ROS production was 10.5-fold increased 5 min after treatment, which decreased to 6.0-fold after 45 min (Fig. 2).

3.3. Gene expression profiling of human monocytes after 45 min ELF-MF exposure

Changes in gene expression of human monocytes were investigated after 45 min ELF-MF exposure using a whole-genome cDNA array Human Unigene RZPD-2. The array was provided with 75,000 clones, of which 38,581 clones including 5,356 annotated genes were expressed.

For analyzing the gene expression alterations, we considered only changes with ≥ 2 or ≤ 0.5-fold, in at least 3 of 4 spots. According to this evaluation protocol, we detected induced alterations in 986 genes, of which 659 were upregulated and 327 were downregulated after 45 min ELF-MF exposure. Genes were subdivided into groups concerning their biological function referring to The Gene Ontology (GO) terms. Some genes are listed in several groups as they contribute to more than one biological function. The following functional categories were chosen: metabolism, cellular physiological processes, signal transduction, response to stimulus, coagulation, extracellular matrix organisation and biogenesis, cell homeostasis, development, oncogenesis, and unknown genes. Nearly 17% of all regulated genes that were detected are with unknown biological functions (Fig. 3A). Major changes in gene expression were found in the metabolism group (45.5%, Fig. 3B), including processes like transcription (FOS, TAZ, DXYS155E, TCF8, HMG20A, COASTER), DNA (PAPOLA) and RNA processing (MGEA6, BICD1, TRNT1, SF3B3), and macromolecule metabolism involving protein modifications (TRIM4, USP2), such as protein dephosphorylation and phosphorylation (SIRT5, PTPN18, ULK2, MLL3, MKPX) (Table 1). Alterations in 27.4% of the total regulated genes were detected in the category of cellular and physiological processes. In this group genes encoding proteins for transport processes (ATP1B2, ENTH, MLL3, NBEA, CHDH, SLC12A8), but also for cell proliferation and cell cycle regulation (MACF1, PDGFA, FHL2, TCF8, FGG, CDC6, MKPX, FHIT) were regulated (Fig. 3B, Table 1).

Our results show that 21.6% of all regulated genes belong to processes which are in the cell communication cluster, including main changes in the signal transduction group (Fig. 3B). These genes mainly indicate regulations of cell surface linked G-protein coupled receptor proteins and intracellular signal cascade proteins (protein kinase C and small GTPases mediated) (data not shown). Gene expression alterations were also detected in MKPX, encoding the mitogen-activated protein kinase phosphatase X, DXYS155E, PDGFA, and GUCY1A3 (Table 1). Furthermore, JAK3, PIAS1, SOCS4, TCF8, involved in the JAK-STAT signalling, and MAPK14, component of the MAPK cascade, were also regulated after ELF-MF exposure (Tables 1 and 2).

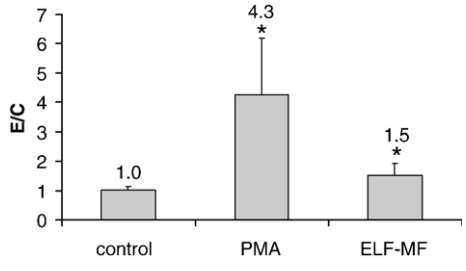

Fig. 1. ROS production in human monocytes after exposure to 1.0 mT ELF-MF or 1 μM PMA for 45 min using the DHR assay. PMA treatment was performed as a positive control for cell activation. The bars represent the arithmetic mean of fluorescence intensity (X-mean) as a ratio of exposed to unexposed cells ± standard deviation (E/C ± S.D.). Cells were derived from 78 donors, pooled and used in 28 independent experiments. Asterisks mark significant differences (Student's *t* test,

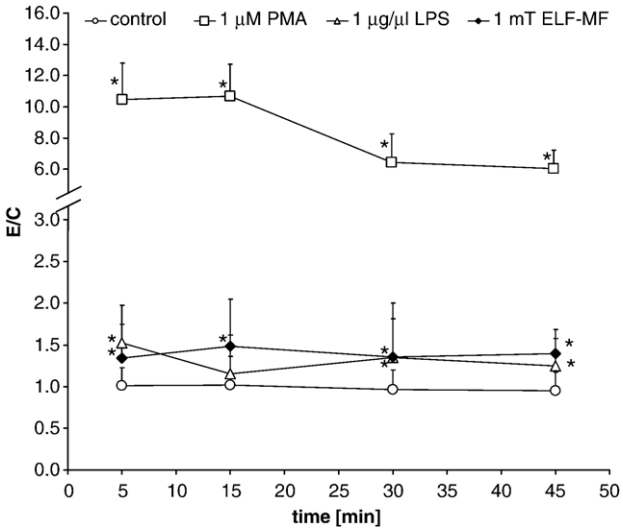

Fig. 2. Time-dependent ROS production in human monocytes after 1.0 mT ELF-MF exposure or treatment with 1 μM PMA or 1 μg/μl LPS for 5, 15, 30 or 45 min. PMA and LPS treatment were performed to induce cell activation (positive controls). The bars represent the arithmetic mean of fluorescence intensity (X-mean) as a ratio of exposed to control cells ($t=0$ min) ± standard deviation ($E/C_{t0} \pm$ S.D.) of 3 independent experiments of 2, 7 and 4 donors. Asterisks mark significant differences (Student's *t* test, $P<0.05$) between

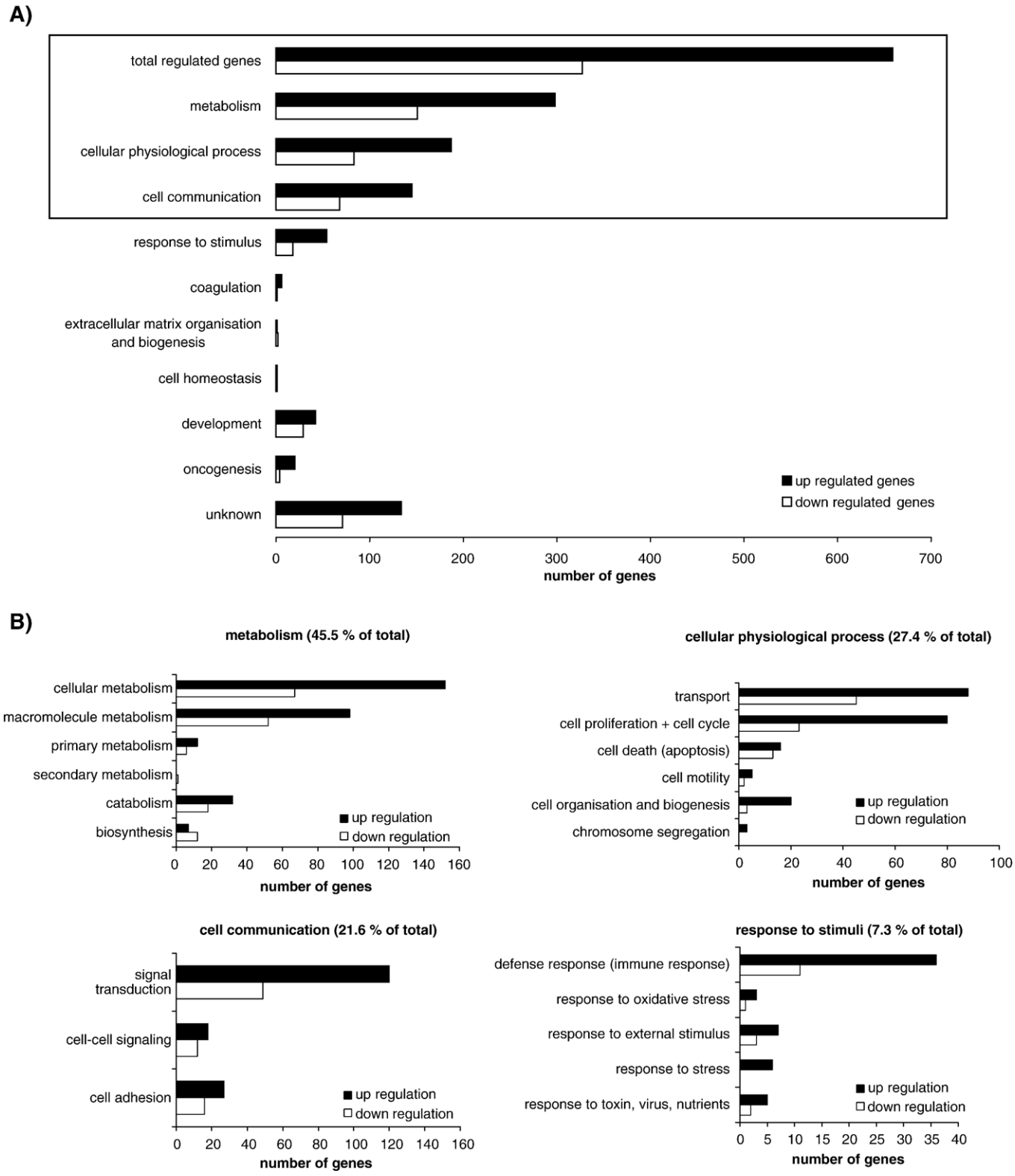

Fig. 3. Alteration in gene expression of human monocytes exposed to 45 min ELF-MF (1.0 mT) using a whole human genome cDNA array including 38,581 genes. Total RNA from 74 donors was isolated and pooled from human monocytes after exposure to ELF-MF or control conditions. Only genes were taken into account, which were at least 2-fold upregulated or downregulated. (A) Upregulated and downregulated genes were subdivided into groups regarding to their biological function: cell communication, cell physiological processes, response to stimuli, metabolisms, coagulation, extra cellular matrix organisation and biogenesis, cell homeostasis, development and oncogenesis. Genes encoding proteins with unknown function are summarised as unknown. (B) The four major groups, metabolism, cellular physiological process, cell communication and response to stimuli, were subdivided into more specific physiological functions. Bars represent the numbers of regulated genes, whereas some genes are mentioned in more than one cluster as they contribute to more than one biological function. Numbers in brackets indicate the percentage of the total 986 regulated genes.

Table 1
Genes with an upregulation (≥5-fold) or downregulation (≤0.2-fold), asterisk marks significant changes of genes with an upregulation of at least ≥2-fold or downregulation at least of ≤0.5-fold, using SAM analysis and a two-tailed *t* test ($P=0.001$), in human monocytes after 45 min ELF-MF exposure

Gene	Cluster description	Mean (ratio)
Down regulation		
ATP1B2	ATPase, Na+/K+ transporting,	0.05
TRIM4	tripartite motif-containing 4	0.07
MGEA6	meningioma expressed antigen 6	0.1
SIRT5	sirtuin 5 (*S. cerevisiae*)	0.2
GUCY1A3	guanylate cyclase 1, alpha 3	0.2
MATN1	matrilin 1, cartilage matrix protein	0.2
PTPN18	protein tyrosine phosphatase, non-receptor type 18	0.2
MGC16121	Hypothetical protein MGC16121	0.4*
DNMT3A	DNA (cytosine-5) methyltransferase 3 alpha	0.5*
Up regulation		
IL20RA	interleukin 20 receptor, alpha	103.9
PSMC2	proteasome (prosome, macropain) 26S subunit, ATPase, 2	71.7
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	56.3
JADE-1	PHD protein Jade-1	33.9
ULK2	unc-51-like kinase 2 (*C. elegans*)	26.1
BICD1	Bicaudal D homolog 1 (*Drosophila*)	19.4
SON	SON DNA binding protein	17.4
TAZ	transcriptional co-activator with PDZ-binding motif (TAZ)	16.3
TRBβ	T cell receptor beta locus	14.5
TRNT1	tRNA nucleotidyl transferase, CCA-adding, 1	13.1
SF3B3	splicing factor 3b, subunit 3	11.5
TOP3A	topoisomerase (DNA) III alpha	10.0
CNP	2-,3-cyclic nucleotide 3- phosphodiesterase	9.5
PAPOLA	poly(A) polymerase alpha	8.1
ENTH	enthoprotin	7.8
DXYS155E	DNA segment on chromosome X and Y (unique) 155 expressed sequence	7.7
MLL3	myeloid/lymphoid or mixed-lineage leukemia3	7.2
MACF1	microtubule-actin crosslinking factor 1	7.0
RINZF	zinc finger protein RINZF	6.9
MORF4L2	mortality factor 4 like 2	6.9
EIF2B3	eukaryotic translation initiation factor 2B	6.5
USP2	ubiquitin specific protease 2	6.3
NBEA	neurobeachin	6.3
PFKFB1	6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 1	6.3
PDGFA	platelet-derived growth factor alpha polypeptide	6.2
CDCA1	cell division cycle associated 1	6.0
TRIM15	tripartite motif-containing 15	5.8
FHL2	four and a half LIM domains 2	5.6
CHDH	choline dehydrogenase	5.5
TCF8	transcription factor 8	5.5
SGT	small glutamine-rich tetratricopeptide repeat (TPR)-containing	5.4
HMG20A	high-mobility group 20A	5.4
FGG	fibrinogen, gamma polypeptide	5.1
CBX5	chromobox homolog 5 (HP1 alpha homolog, *Drosophila*)	5.1
COASTER	coactivator for steroid receptors	5.1
CDC6	CDC6 cell division cycle 6 homolog (*S. cerevisiae*)	5.1
MKPX	mitogen-activated protein kinase phosphatase x	5.1
FHIT	fragile histidine triad gene	5.0
SLC12A8	solute carrier family 12, member 8	5.0
DKFZP586J1624	Unknown	6.3*
EPS15R	Epidermal growth factor receptor pathway substrate 15 - like 1	2.8*
IL15RA	Interleukin 15 receptor, alpha chain	2.6*

The category response to stimulus includes 7.3% of total regulated genes after ELF-MF exposure (Fig. 3B). Within this cluster, main changes were detected in the category of defence response including genes for regulation of the immune response, such as FOS, TRB, DXYS155E, and TCF8. Genes for the IL-20 receptor alpha chain (IL20RA) and cyclic nucleotide phosphodiesterase (CNP), which, respectively, signal responses to stress (defence response), were also regulated (Table 1). Downregulations for the chemokine receptor CCL19 and CCL27 and upregulations for the immune responding genes IL-1F5 and C3, indicate activation of immune cells (Table 2).

A further evaluation of data using the Statistical Analysis of Micro array (SAM) was applied, where clones with 4 good quality spots were taken into consideration. According to this evaluation protocol, 36 significantly upregulated and 51 downregulated genes were detected (data not shown). All these clones resulted also in significant changes with respect to a two-tailed *t* test with $P=0.001$. If regulation of ≥2-fold or ≤0.5-fold were taken into consideration, only 12 upregulated and 31 downregulated genes could be detected. Only 4–5 genes are belonging to known genes, whereas significant gene alterations were detected mainly in, so far, unknown genes. The DKFZP586J1624 (6.3-fold), EPS15R (2.8-fold), and IL15RA (2.6-fold) genes were upregulated, while MGC16121 (0.4-fold), and DNMT3A (0.5-fold) were

Table 2
Genes with an upregulation (≥2-fold) or downregulation (≤0.5-fold) in human monocytes after 45 min ELF-MF exposure regarding to the gene ontology group immune response

Gene	Cluster description	Mean (ratio)
Down regulation		
CCL27	chemokine (C–C motif) ligand 27	0.3
CCL19	chemokine (C–C motif) ligand 19	0.4
Up regulation		
PIASY	protein inhibitor of activated STAT protein PIASy	2.7
MAPK14	mitogen-activated protein kinase 14	2.7
SOCS4	suppressor of cytokine signalling 4	2.6
C3	complement component 3	2.3
IL1F5	interleukin 1 family, member 5 (delta)	2.2
JAK3	Janus kinase 3 (a protein tyrosine kinase, leukocyte)	2.1

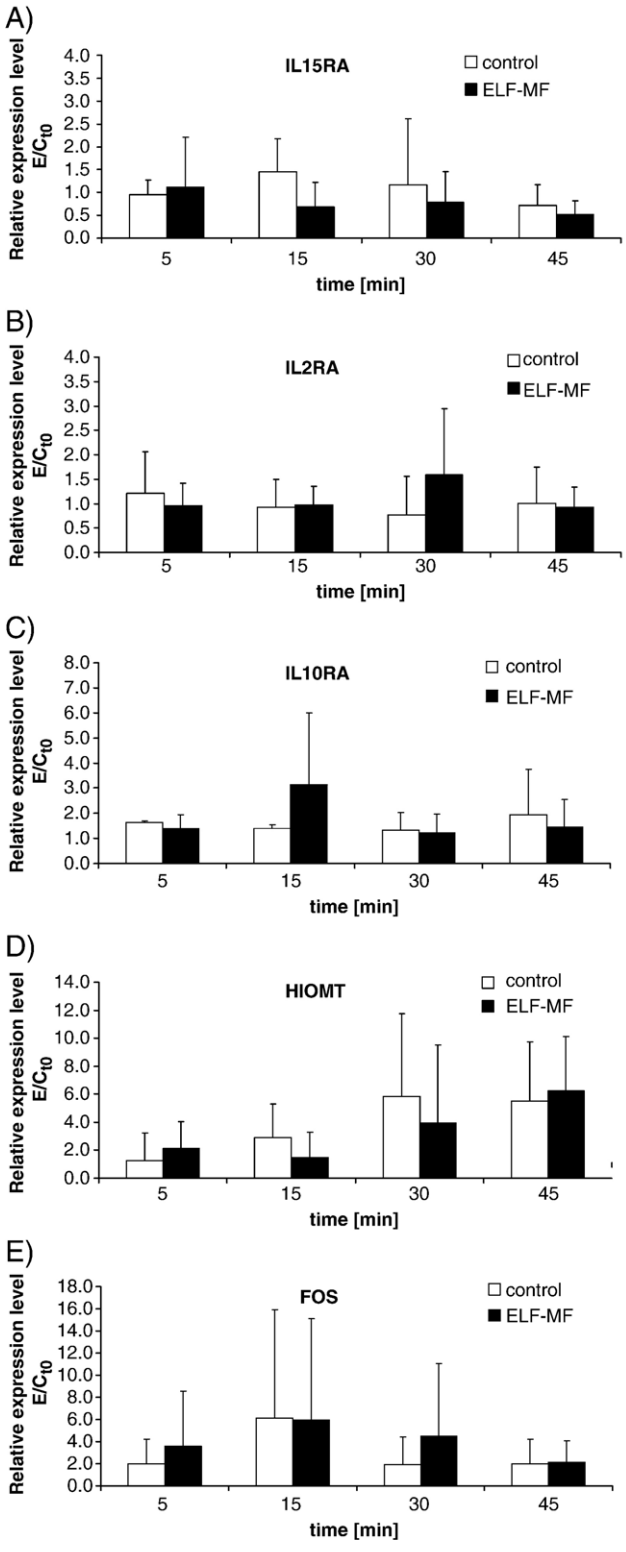

Fig. 4. Changes of gene expression for IL15RA (A), IL2RA (B), IL10RA (C), HIOMT (D) and FOS (E) after 5, 15, 30, 45 min ELF-MF exposure in human monocytes; performed by using the real-time RT-PCR. Data represent the arithmetic mean ratio to control (t=0 min)±standard deviation (E/C_{t0}±S.D.) of 2 independent experiments carried out in duplicates. Values were normalized to hypoxanthine phosphoribosyl-transferase1 (HPRT) and ratios were calculated

downregulated (Table 1). The gene IL15RA, encodes the α chain of the IL-15 receptor and acting as a regulatory factor during inflammation was significantly upregulated. EPS15R is related to endocytosis and DNMT3A is involved in DNA methylation which could be a good link to cell activation processes, while the biological function of DKFZP586J1624 and MGC16121 is not clear yet.

3.4. Kinetics of gene expression

Gene expression profiling showed a significant change of IL15RA regulation, encoding the cytokine receptor α chain for IL-15. Therefore, we investigated the IL15RA expression during 45 min ELF-MF exposure using real-time RT-PCR. In addition, we also studied the IL2RA expression, since both IL15RA and IL2RA share the β and γ chain of the IL-2 receptor. They hereby contribute to similar signalling cascades in the pro-inflammatory immune response. IL10RA encoding the α chain for IL-10 receptor is also involved in the immune response by representing an anti-inflammatory function, therefore IL10RA gene expression was also studied.

The kinetics of the gene expression showed a divergent regulation of the 3 cytokine receptors IL15RA, IL2RA, and IL10RA in human monocytes during 45 min ELF-MF exposure (Fig. 4A–C). The upregulation of IL10RA and a downregulation of IL15RA were already induced after 15 min ELF-MF exposure, whereas no regulation of IL2RA was observed at that time point. The regulation of IL10RA and IL15RA reached the control level after 30 min, whereas an upregulation could be observed for IL2RA, which was decreased to the control level after 45 min.

The early response gene FOS was upregulated whereas the melatonin synthesizing enzyme HIOMT was not regulated in the gene expression profiling. Since their encoded proteins are involved in the immune response we studied the gene expression kinetics for these genes as well. Interestingly, HIOMT was downregulated for the first 30 min, but reached the control level again after 45 min exposure (Fig. 4D) confirming the array result. The FOS encoding gene was not regulated for the first 15 min, but we detected an elevated expression after 30 min exposure, which was regulated towards the control level after 45 min (Fig. 4E), not identical to the array data. All data were compared to the control value t=0 min. It should be pointed out that the RT-PCR studies were carried out in two independent experiments using different donors than for the array screening, which could be the reason for the inconsistent results.

4. Discussion

This study focused on the capacity of ELF-MF to activate human umbilical cord blood-derived monocytes resulting in an increased release of free radicals. Gene regulation in ELF-MF exposed monocytes was investigated to achieve a general view of involved activating pathway(s). However, previous studies have already shown the activating capacity of ELF-MF in murine macrophages, human Mono Mac 6 cells and human monocytes by an increased free radical production [1,3], as well

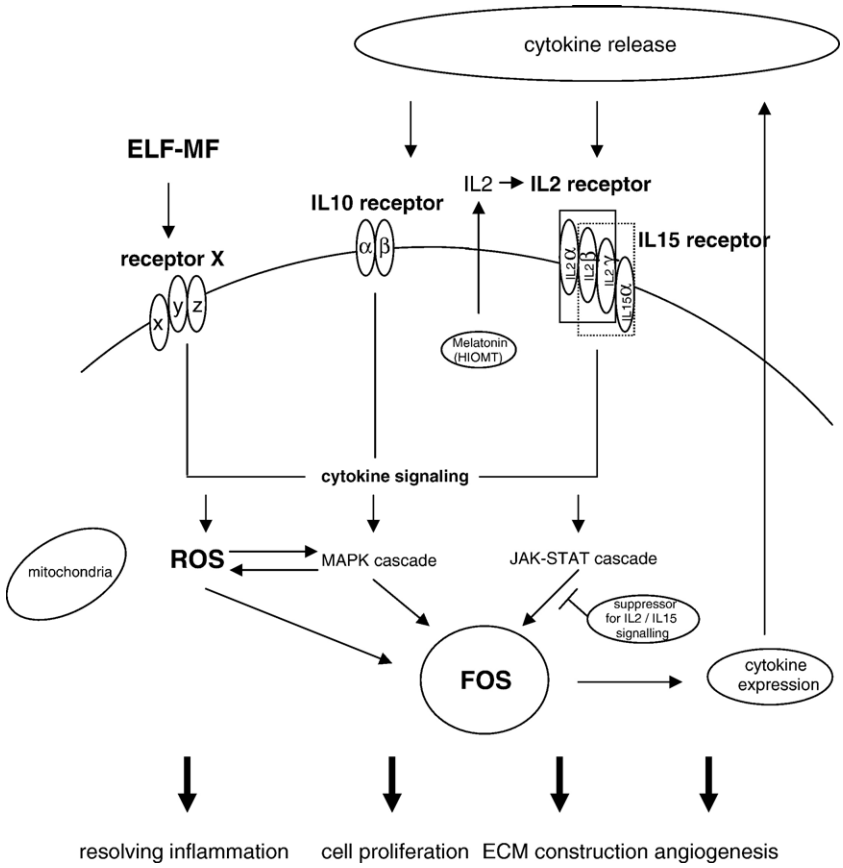

Fig. 5. Induction of the alternative activation pathway in human monocytes during 1.0 mT ELF-MF exposure. Cell activating capacity was immediately detected as an increased ROS production after ELF-MF exposure, where we assume that the cell activation is mediated via a non-identified receptor-dependent pathway. Subsequently, ROS initiates the expression of cytokine signalling by an increased expression of the transcription factor FOS, resulting in an increased cytokine release causing the expression of the cytokine receptor IL15RA and IL2RA – two pro-inflammatory cytokine receptors – and IL10RA, indicating an anti-inflammatory response. Thus, cytokine signalling maintains ROS release and FOS expression via the MAPK cascade, while JAK-STAT cascade via IL2 and IL15 receptor might be suppressed by the decreased melatonin production, indicated by a decreased expression of the HIOMT gene, and by the expression of genes encoding proteins for inhibiting IL-2 production (e.g., TCF8) and JAK-STAT signalling (SOCS4). Therefore, we assume that no inflammation is induced by ELF-MF. Furthermore, gene expression profiling and gene kinetic analysis confirm that inflammation is resolved and cell proliferation, ECM construction, and angiogenesis are promoted, indicating the involvement of the alternative pathway.

as by an increased phagocytotic uptake [2], and IL1β production [29] in murine macrophages.

Our results confirm the cell activating capacity of ELF-MF in human monocytes and emphasize the influence of ELF-MF on the used immune cells by a significant increased ROS release already after 5 min ELF-MF exposure, which remained constant over the observed time period (45 min). Furthermore, our results confirm the assumption that ELF-MF activates monocytes via a receptor-dependent pathway similar to LPS, since the ELF-MF induced ROS release was detected at the same magnitude as LPS. It was shown that the LPS receptor encoding gene is 2-fold upregulated after cell activation in the gene expression profiling [30], which is initiated when LPS-binding protein (LBP)3 transfers LPS to CD14 on the surface of cells. LPS-CD14 complexes then signal via Toll-like receptors to activate NF-κB as well as the extracellular regulated kinase, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases, all of which mediate the production of inflammatory cytokines [30].

In contrast, PMA treatment induces cell activation via a receptor independent pathway, causing a ROS release initiating the induction of an oxidative burst. The rise of the intracellular oxidant level produced by ELF-MF, PMA or LPS, can result in two potentially important effects: (i) damage to various cell components and triggering the activation of specific signalling pathways [4], and (ii) ROS play an important role as second messenger involved in downstream signalling, resulting in turning on genes with important functions in the immune response and/or cellular physiological processes such as cell proliferation [6,31].

To obtain an indication of gene regulation after 45-min ELF-MF exposure of monocytes, gene expression profiling was carried out. The gene expression pattern of the pooled RNA of 78 donors shows regulation of 986 genes after only one time point, namely after 45 min ELF-MF exposure. Our result shows alteration of signal transduction and immune response related genes, although the main changes were detected of genes referring to metabolism and cellular physiological processes.

Statistical analysis indicated the significant regulation of 5 annotated genes (DKFZP586J1624, EPS15R, IL15RA, MGC16121, and DNMT3A) with 4 good quality spots. Since we

were interested in receptor-dependent regulation, we investigated IL15RA, encoding the α chain of the interleukin receptor for IL-15. IL-15 is a cytokine and structurally related to IL-2, eliciting similar biological effects in vitro, explained by the common usage of the IL-2 β-γ signalling complex. Both cytokines trigger similar downstream signalling pathways including the activation of Jak-1/Jak-3 tyrosine kinases, Lck and Syk tyrosine kinases, the MAPK pathways, and induction of Bcl-2 [16,17].

Our results on gene expression profiling show (see Tables 1 and 2) the upregulation of JAK3 and MAPK14, encoding proteins involved in the JAK-STAT or MAPK signalling pathway. JAK-STAT cascade suppressing genes such as PIAS1, SOCS4 and TCF8, an IL-2 suppressor were also regulated at the observed time. However, the activation of the MAPK pathway seems to initialize the activation of small GTPases, which are molecular switches that use a simple biochemical strategy to control complex cellular processes. Various small GTPases were upregulated, mainly belonging to the major groups Ras, Rho (Rac), and Rab. One of the most interesting aspects of these regulatory GTPases is that they also regulate a variety of biochemical pathways including MAPK pathway, and the phagocytotic NADPH oxidase pathway [32].

A common effect of the activation of signal transduction pathways, e.g., MAPK pathway or NADPH pathway, is the modulation of transcription factors such as FOS. FOS can also be regulated by ROS, initiating gene expression important for immune response [31,33], and also implicated in the regulation of cell proliferation [34]. FOS activation has been reported by other research groups after ELF-MF exposure as well [19–21]. Therefore, we assume that FOS plays a central role during ELF-MF exposure, since our results of the gene expression profiling show more upregulations than downregulations in the group "response to stimulus (immune response)". Additionally, upregulation of various genes regarding cell proliferation (e.g., platelet-derived growth factor alpha polypeptide—PDGFA) [35] and angiogenesis (e.g., vascular epidermal growth factor—VEGF) [36] were also detected.

Taken together, the array data indicate that ELF-MF activate human monocytes directly or indirectly via the alternative pathway. Consequently, inflammation is not induced, shown also by the significant upregulation of IL15RA and SOCS4 as well as TCF8, which are suppressors of interleukin signalling. Our assumption is furthermore supported by the downregulation of chemokine genes CCL19 and CCL27, usually involved in inflammatory response. di Luzio and co-workers have described that the two inflammatory chemokines MCP-1 and RANTES were downregulated in human monocytes exposed to ELF-MF (50 Hz, 1.0 mT) for 24 h at the protein level [37]. In addition, initiation of IL-1F3 release [38] counteracts inflammatory processes [39]. Our result in the gene expression profiling showed an upregulation of IL-1F5, which is closely phylogenetically related to IL-1F3 [40].

To elucidate the involvement of the alternative pathway during the activation of ELF-MF exposed monocytes, the kinetics of the IL15RA gene was analyzed by real-time RT-PCR. Furthermore, IL2RA was investigated, since both IL15RA and IL2RA indicate pro-inflammatory responses. IL10RA was also examined for gene expression as it is involved in the anti-inflammatory response. The gene encoding HIOMT and the FOS gene, which are involved in the immune response, were also observed to obtain more information of cell activation processes during 45 min ELF-MF exposure. Our results show the upregulation of IL10RA after 15 min, mediating the immunosuppressive signal of IL-10 and inhibiting the synthesis of pro-inflammatory cytokines [28]. IL15RA regulating pro- inflammatory processes, was already downregulated at that time point, demonstrating the repression of inflammatory response. IL2RA expression, which is associated with adaptive immunity, was not regulated at 15 min, but it showed an upregulation at 30 min. It has been shown that monocytes do express IL2RA on their cell surface resulting in an increase of ROS [41]. The downregulation of IL2RA at the end of exposure to the control level can be explained by the increased expression of the IL-2 suppressor TCF8 shown in the array analysis, which inhibits the IL-2 production and hereby the IL2RA expression [42,43].

Besides, HIOMT encoding an enzyme involved in melatonin synthesis [44], and in the increase in IL-2 production [18], was downregulated during ELF-MF exposure, as shown with the RT-PCR analysis. Some studies have shown that ELF-MF inhibited the production of melatonin and the authors linked a low level of melatonin to breast cancer and other tumour development (for review, see [45]). One of the functions of melatonin is the action as a receptor independent free radical scavenger. Downregulation of melatonin synthesizing enzymes can cause a lack of free radical scavenger in a biological system. A recently published study showed that melatonin is synthesized in human lymphocytes, and is therefore involved in inflammatory processes [18]. A downregulation of melatonin synthesis enzymes such as HIOMT could be an indication of the absence of acute inflammatory processes.

FOS expression seems to be activated by ROS, which is immediately induced by ELF-MF via a receptor-dependent pathway, although the receptor has not been identified yet. Consequently, the induction of FOS leads to cytokine release and to the expression of their cytokine receptors, leading to the activation of a cytokine signalling pathway, causing the modulation of the activities of transcription factors such as FOS [4,9]. Cytokine signalling is also maintained by the production of ROS via the MAPK cascade, affecting the FOS expression. The JAK-STAT signalling induced by IL2 and IL15 receptor seems to be inhibited by specific suppressors and possibly by a decreased melatonin production (Fig. 5).

Our results from the gene expression profiling simply represent the average of 78 donors at one time point during cell activation, while the investigation of real-time RT-PCR analysis displays the average of gene expression of two independent experiments, derived from 5 and 6 different donors, respectively. The individuality of each experiment explains the wide standard deviation of the gene expression investigations during 45 min ELF-MF exposure in monocytes. We do not expect any improvement of the data set by several repetitions, because of the different donors. Therefore, no statistical analysis could be performed for real-time RT-PCR data. However, the

present study shows upregulation or downregulation tendencies, which are discussed in the present paper.

In summary, we showed the cell activating capacity of ELF-MF in human monocytes for the release of ROS. Alterations in gene expression and real-time RT-PCR analysis of IL15RA, IL2RA, IL10RA, HIOMT and FOS confirmed the assumption that ELF-MF activates human umbilical cord blood-derived monocytes directly or indirectly via the alternative pathway. Further investigations have to be performed to confirm this assumption.

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